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Performance of CD3xCD19 Bispecific Monoclonal Antibodies in B Cell Malignancy

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Bispecific monoclonal antibodies, with a dual specificity for tumor associated antigens on target cells and for surface markers on immune effector cells, have been shown (in vitro) to be effective in directing and triggering effector cells to kill target cells resulting in target cell lysis. Bispecific monoclonal antibodies (BsAb) against the CD3 antigen on T cells and the CD19 antigen on B cell were developed. Data obtained by in vitro experiments might indicate that clinical responses in BsAb immunotherapy, will only be obtained in patients with minimal tumor load, and may need additional T cell stimulation via cytokines such as IL-2. Although these experiments have shown us their limitations, they also include the promise of BsAb-directed immunotherapy in B cell malignancy as further demonstrated during a Phase I trail, showing little toxicity. Clearly, much remains to be done before this BsAb is routinely used for therapy, but, the results presented show that the CD3xCD19 BsAb has a potential as a therapeutic agent in B cell malignancy. This report describes the experiments performed to test a new immunotherapeutic approach for the treatment of B cell malignancy. Bispecific antibodies are described that can target cytotoxic T cells to tumor cells and elicit a cytolytic action towards these cancer cells.

KEY WORDS: immunotherapy bispecific antibodies B cell malignancy T cell activation

INTRODUCTION

Chemotherapeutic treatment of advanced B cell malignancies can only cure a proportion of patients, although progression has been noted in the last 40 years since the first described active cytostatic drug. Even with modern aggressive treatment, 30% to 60% of the patients have resistant disease or relapse,[1] indicating that development of other therapies than chemotherapy and radiotherapy seems worthwhile.[2,3]

Immunotherapy may consist of the stimulation of host anti-tumor immunity or the administration of biologically active agents with innate anti-tumor properties. The administration of several biological response modifiers, including interferons (IFN), interleukins (IL), tumor necrosis factor, and hematopoietic growth factors may upregulate host immunity (IL-2) or induces direct anti-tumor activity (TNF, IFN).[4] Some durable complete responses have been reported with these agents, but substantial toxic effects have been found as well, often life threatening, at doses required to achieve durable complete responses.[2,4–7] Cellular adoptive therapy i.e. administration of cell subsets with anti-tumor effector functions, involves a variety of immune cells including in vitro activated T cells (LAK, lymphokine activated killer cells; TIL, tumor infiltrating lymphocytes) and monocytes. The therapeutic efficacy of the cells in vivo may be hampered by several limitations related to the effector-cell population used. For example, LAK cells have a limited ability to traffic to certain tumor sites[8–10] and need IL-2 in order to maintain their anti-tumor activity.[11] TILs have a higher anti-tumor activity, but cannot always be isolated.[2,12,13]

Monoclonal antibodies (mAb) directed against certain antigens on tumor cells offer the possibility of a specific (passive) therapy of malignant disease. Unconjugated mAb achieved anti-tumor activity by physiological ef-

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fector mechanisms e.g. directing antibody-dependent cellular cytotoxicity or complement mediated lysis. Although cytotoxicity is clearly demonstrated in vitro, the expected results in vivo are disappointing. Monoclonal antibodies used in the treatment of tumors showed only a few sustained responses.[2,14,15] Immunotoxins (monoclonal antibody-toxin conjugates) are a class of immunopharmacologic agents that show considerable possibilities for more effective treatment[14,16,17] and are currently being tested in phase I and II trails. This holds true for radio-immuno-conjugates as well.[14,18,19]

A new form of immunotherapy for the treatment of cancer (and infectious diseases) has recently been developed that uses bispecific monoclonal antibodies (BsAb) to redirect effector cells to target cells. Since Köhler and Milstein demonstrated, in 1975, that individual clones of normal antibody secreting cells could be immortalized by fusion with myeloma cells,[20] virtually unlimited quantities of homogenous, exquisitely specific antibodies can now be produced.[21,22] New developments in hybridoma technology have enabled the production of hybrid hybridomas, which harbour the genetic information of both parental hybridoma clones. From the produced Ig species, bispecific antibodies can be isolated. These BsAb may be designed to link target cells to cytotoxic trigger molecules, like T cell receptors or Fcγ receptors (FcγR) on cytotoxic cells and thus induce target cell lysis. Because this therapeutic approach uses normal cellular immune defense mechanisms, it may lack the toxicity associated with immunoconjugates.[23] Apart from hybrid hybridoma derived BsAb, discussed below more extensively, prior methods included chemical cross-linking[24–32] and recently molecular genetic approaches[33–36] have been developed.

BsAb in redirected cytotoxicity involves binding of the BsAb to both the target cell (tumor cell or pathogen) and cytotoxic trigger molecules (TCR/CD3 or Fcγ receptor) on the effector cell. The effector cell then kills the target cell irrespective of its intrinsic specificity (Fig. 1). Since the first reports in 1985,[25,37] describing the in vitro efficacy of BsAb in redirected cytotoxicity, results have been promising (reviewed in[38–42]). BsAb mediated therapy in vivo has several requirements: 1) recruitment of sufficient effector cells and effector cell activation; 2) high antigen affinity, to induce a stable adhesion between effector and target cells, which is necessary because of monovalent binding (which minimizes modulation of the antigen[43,44]); 3) high level of specific killing of the desired target and no reciprocal killing of the effector cell. BsAb have been found to exert two different anti-tumor effects, a direct lytic effect (via attachment of target and effector cells), and a tumor growth inhibiting effect (lymphocytes secreting cytokines upon targeting).[42] BsAb in vitro have been tested for the killing of a wide variety of tumor cells e.g. carcinoma cells,[45] glioma cells,[46] lymphoma cells[47–49] or myeloid cells.[50]

The in vivo efficacy of BsAb mediated tumor cell killing, has been demonstrated in murine models using (intact) BsAb.[51–53] Several clinical trials have been reported.[41,50,54–57] In two trials patients were treated locoregionally with BsAb and ex vivo activated T cells, with sometimes favorable results.[54,55] Sofar a preliminary trial of a CD3xCD19 BsAb in one patient with B cell malignancy has been reported.[58] In this review, various aspects of the potential of CD3xCD19 BsAb in B cell malignancies are explored. In a clonogenic assay the importance of repeated addition of BsAb with IL-2 to obtain maximal tumor cell killing was studied. This combination of factors leads to optimal T cell activation and might indicate that ex vivo activation of T cells is not necessary. The significance of T cell activation in vivo is made clear by the fact that in vitro activated T cell have a different homing pattern when reinjected in to the patient. Further, the importance of T cell activation in vivo restricted to the tumor site will be discussed.

The target cell antigen: CD19

Monoclonal antibodies directed against surface antigens on a wide variety of cell types are being increasingly explored for the therapy of cancer (reviewed in[14,59]). The Cluster of Differentiation 19 (CD19) defined antigen is a B cell-specific molecule expressed on virtually all cells of the B cell lineage except on plasma cells. CD19 antigens, together with CD22 and B7 (CD80) antigens, are members

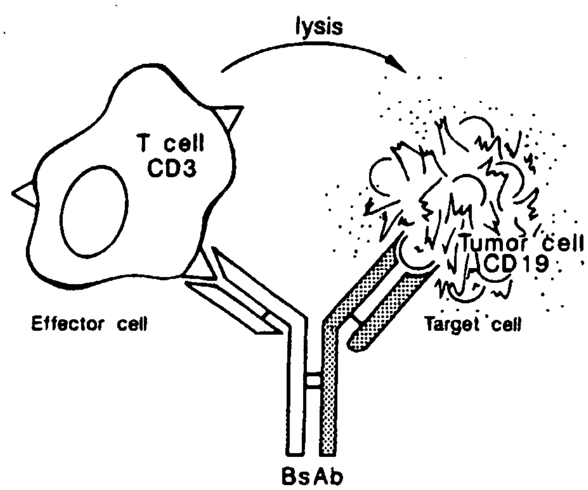

Figure 1 Drawing illustrating targeted lysis. Cytotoxic T cells are redirected to lyse tumor-target cells through bispecific monoclonal antibodies.

of the Ig supergene family.[60,61] It is a component of a complex that contains at least two other membrane proteins, complement receptor type 2 (CD21/CR2) and TAPA-1 (CD81).[62] Its expression precedes all other B cell restricted antigens in early B cell ontogeny, and is expressed before Ig Heavy (H) chain rearrangement. CD19 can act as a signal-transducing molecule involved in the regulation of B cell proliferation and terminal B cell differentiation.[63] CD19 can deliver either a positive or a negative signal, depending on both the state of B cell differentiation and the way in which the B cells are activated.[62,64–66]

CD19 antigen is expressed on leukemic B lineage lymphoid progenitor cells but not on myeloid, erythroid, megakaryocytic, or multilineage bone marrow progenitor cells. CD19 mAb show no reactivity with T cells, granulocytes, RBCs, or platelets. CD19 mAb do not react with normal non-lymphohematopoietic tissues.[17,67,68]

These characteristics led to the use of CD19 mAb for immunotherapy either as unconjugated mAb,[69,70] or as a carrier of toxins.[66,71] Administration of unconjugated CD19 mAb (mIgG2a) to six patients with progressive B cell non-Hodgkin's lymphoma (NHL) induced tumor regression in two patients but responses were limited and of short duration.[70] However, during CD19 mAb therapy it was noticed that free CD19 antigen could not be detected in the serum. Further, although CD19 mAb can induce modulation of the antigen in vitro,[72,73] complete modulation of CD19 antigen on tumor cells has not been found in vivo. Also, treatment with CD19 mAb induced no toxicity (doses of 250 mg given i.v.), and none of the patients in that study or a follow up study developed antibodies against mouse immunoglobulin.[2,69,70] A potential disadvantage is that also normal B cells can be killed by the CD3xCD19 BsAb treatment. However, B cells will be rapidly replaced by differentiation from stem cells and antibody/immunoglobulin levels will not be seriously affected as plasma cells lack CD19. These findings implicate that the CD19 antigen may be a good candidate for BsAb mediated tumor cell targeting.

The effector cell antigen: CD3

Cellular adoptive immunotherapy of cancer can be performed using different cytolytic effector cells e.g. T cells, NK cells, monocytes and macrophages. Several surface molecules involved in triggering (the lytic) activation of T cells have been identified; TCR/CD3, CD2 and CD28. CD3 mAb are most widely used to activate T cells.

The T cell receptor (TCR) is a multi-subunit complex responsible for recognition of a specific antigen in the context of a particular MHC product by a given antigen presenting cell. The signal transduction capacity of the CD3 unit is expressed in the extensive cytoplasmic tails characteristic for each of the individual proteins.[74] In vivo T cell activation requires, in addition to this antigen-specific signal, a co-stimulatory signal delivered by accessory receptors following their engagement by ligands expressed on antigen-presenting cells.[75] In vitro, this antigen-specific signal can be mimicked by cross-linking of the TCR complex via Ab to the antigen receptor component, but especially by CD3 mAb. This signal then initiates a series of intracellular events that ultimately lead to expression of interleukin-2 receptors, lymphokine production, and entry into the cell growth cycle. To obtain sufficient cytotoxic T cells (lymphokine activated killer cells) for adoptive immunotherapy, peripheral blood lymphocytes (PBL) can be activated with such mAb or with the lectin phytohemagglutinin (PHA), in either case combined with IL-2.[76,77] Large scale expansion can also be performed using the same activators with lymphocytes isolated from local tumor sites (tumor infiltrating lymphocytes).[13]

The use of BsAb for redirected killing by T cells has recently been reviewed.[41] T cells are readily available and BsAb mediated killing through the TCR/CD3 complex is not MHC restricted and may involve both $CD4^+$ and $CD8^+$ cells.

Here we describe the development and testing of bispecific antibodies, that can be used for immunotherapy in patients with B cell malignancy. Based on the considerations mentioned before it was chosen to focus on a CD3xCD19 BsAb as immunotherapy in patients with B cell malignancy.

FcγR dependent T cell activation by CD3xCD19 BsAb

Bispecific monoclonal antibody (BsAb) therapy may be performed with biologically produced intact Ig molecules[40] and may be used to influence the immunological status of the patient.[14,59] Different (sub-)classes of murine and rat antibodies (Ab) are known to have variable ability to interact with human complement and/or Fcγ receptors on effector cells.[78,79]

The H chain isotype composition will, thereby, influence the ability of the BsAb to interact with Fcγ receptor positive cells. Such Ab interactions might lead to negative side effects as a result of (increased) cytokine release.[80–83] These FcγR interactions may be required to result in favorable T cell activation,[84–86] which is a prerequisite for efficient killing of tumor cells upon targeting by the BsAb.

To be able to compare positive and negative contributions of FcγR interactions with BsAb in vitro and in vivo, we constructed two hybrid hybridomas, QAI-2 and QAI-3, each producing a BsAb with identical binding sites for

the CD3 and CD19 antigen, but differing in isotype composition of their Fc domain. The hybrid hybridomas were produced as reported earlier,[29] by using PEG-mediated fusion of a HAT^{s}/neo^{r} cell line with a "wild-type" line. Both BsAb were selected by testing the supernatant of producing clones in double isotype ELISA (DIE)[29,87] and cytotoxicity assays. Purification of the BsAb was performed on an HPLC-ABx column. The A_{280} profiles of QAI-2 and QAI-3, showed only three symmetrical peaks with clear intervals, suggesting preferential homologous H and L chain pairing.[31] The first BsAb was derived from mouse (m) IgG1 and mIgG2a mAb, while in the second BsAb the mIgG2a was replaced by the mIgG2b isotype. These two BsAb were compared with a third CD3xCD19 BsAb; the SHR-1 (ratIgG2bxmIgG1).[88] Parental mAb and the composition of the BsAb are detailed in Table 1. All three purified BsAb proved equally effective for their ability to target pre-activated T cells towards CD19 positive tumor cells.[89]

Three major classes of leukocyte IgG Fc receptors are currently recognized; all have been mapped to the long arm of chromosome one.[78,90] Human monocytes express FcγRIa (CD64) and FcγRIIa (CD32).[91] The FcγRIIa-R131, with Arginine at position 131 was previously named "high responder" because of its high reactivity with mIgG1, in contrast to the FcγRIIa-H131 (with Histidine at position 131 and was previously named "low responder"). In T cell proliferation assays, the role of these purified BsAb was evaluated, and the capacity of FcγRIa (CD64), FcγRIIa-R131 and FcγRIIa-H131 (CD32) transfected fibroblasts to present the BsAb was tested.[89,92]

MouseIgG1-IgG2a BsAb/QAI-2 did induce T cell activation when presented by FcγRIa transfectants, extending our previous findings in antibody-dependent cellular cytotoxicity (ADCC) experiments.[93] Apparently, the presence of only one mIgG2a H chain in an Fc domain suffices for binding. Also in line with the ADCC results, the mIgG1-mIgG2b BsAb/QAI-3 did not induce a significant T cell proliferation after interaction with the FcγRIIa-R131 transfectant. BsAb mIgG1-mIgG2a QAI-2 stimulates T cell proliferation to the same extent as seen with parental CD3 mAb, despite its monovalency for the CD3 antigen. Therefore, FcγR interaction of this mAb may well serve to activate the T cell as a preliminary step in targeting cellular cytotoxicity. Although both BsAb bind to CD3 and CD19 antigens, they did not induce T cell proliferation in the presence of several CD19 positive tumor cell lines, but did so when autologous monocytes were added.[89] The Fc domain thus seemed a necessity for T cell activation. The third BsAb, SHR-1 (CD3-ratIgG2b × CD19-mouseIgG1), was like the mIgG1xmIgG2a BsAb/QAI-2 only capable of binding FcγRI.

However, cross-linking of FcγRIa on monocytes may at the same time trigger them to produce cytokines. These cytokines (i.e. IL-6 and TNF-α) are probably responsible for the severe side effects seen in mAb treatment.[80–83,94] The mIgG1-mIgG2b BsAb/QAI-3 could not induce T cell mitogenesis and is therefore most likely not able to bind to the examined FcγR and is thus not expected to induce cytokine release by accessory cells. For effective use of this BsAb additional signalling by IL-2 (or CD28 mAb) might be needed.

Therefore, BsAb with functionally different Fc domains represent alternative strategies in BsAb therapy, the efficacy of which deserves to be compared in vivo.

Table 1 Parental and Bispecific mAb

Parental Ab	*Specificity*	*Isotype*	*Reference*
CLB-T3/4.1	CD3	mouse IgG1	(148)
CLB-T3/4.2a	CD3	mouse IgG2a	(148)
CLB-T3/4.2b	CD3	mouse IgG2b	(148)
SHL45.6	CD3	rat IgG2b	(88)
CLB-CD19	CD19	mouse IgG2a	(73)
CLB-CD19	CD19	mouse IgG2b	(89)
BsAb	*Specificity (isotype)*		*Reference*
QAI-2	CLB-T3/4(mIgG1) × CLB-CD19(mIG2a)		(89)
QAI-3	CLB-T3/4(mIgG1) × CLB-CD19(mIG2b)		(89)
SHR-1	SHL45.6(rIgG2b) × MG1CD19(mIgG1)		(88)

Killing of autologous tumor B cells using CD3xCD19 BsAb

The in vitro efficacy of the ratIgG2bxmouseIgG1 BsAb/SHR-1, directed against the T cell antigen CD3 and the B cell antigen CD19, to induce (malignant) B cell kill by activated human T cells was first measured in a ^{51}Cr-release assay.[47,95] The concentration of the BsAb and the effector to target cell ratio to induce optimal tumor cell lysis, were tested using two cytotoxic T cell clones (TCRαβ and TCRγδ) against CD19 positive target cell lines. In all cases 100 ng/ml appeared to be sufficient to generate optimal induction of lysis of the CD19^{+} cell lines used. In later experiments, freshly isolated malignant B cells were used. These target cells, independent of the diagnosis and the source of the target cells, were efficiently killed by BsAb-preincubated CTL clones as well as by phytohaemagglutinin or CD3-activated IL-2 expanded peripheral blood mononuclear cells. Efficient cytolytic activity was obtained even by low effector to target cell ratio's (9:1). The killing mediated by the BsAb was highly specific since CD19 negative cells ("bystander" cells) were not killed. No induction of lysis was seen with both parental mAb i.e. CD3-rIgG2b or CD19-mIgG1, when used in a concentration of 1 μg/ml.[47]

Prior to clinical application in a phase I study, PBMC obtained from patients in different stages of their disease

have to be tested for their suitability of activation. End stage leukemia/lymphoma patients are immunocompromised and the number and activation status of the T cells might be insufficient. Therefore, we extended our study by the use of patient-T cells, activated in vitro by CD3 mAb and IL-2. PBMC isolated from patients with NHL or acute lymphoblastic leukemia (ALL) during remission or relapse rapidly proliferated on in vitro activation (PHA or CD3 mAb with IL-2). During those three weeks of stimulation the phenotype of the PBMC changed into predominantly (>90%) $CD3^+/CD8^+$ cells. Those activated T cells were equally effective in killing cells from B cell lines as the T cell clones. Moreover, they showed the same performance as the T cell clones towards autologous tumor cells. In addition, these activities of patient derived effector cells proved independent of the remission or relapse phase of the disease. Although in naturally occurring T cell-mediated cytotoxicity, a number of membrane molecules present on effector and/or target cells may contribute to the effectiveness of killing,[96–102] we found BsAb-mediated tumor cell lysis to be independent on the expression of HLA class I,[103] accessory molecules (CD11a/LFA-1 and CD54/ICAM-1[98,104–106]), costimulatory molecules (CD80/B7)[107,108] and resistance to biological response modifiers (TNFα, IFNγ).[2,109,110] The presence of the CD3xCD19 BsAb was absolutely required for lysis of autologous tumor cells.

Until this stage we used preactivated T cells in BsAb mediated tumor cell killing. An important question is whether resting PBL-T cells are also activated when this CD3xCD19 BsAb is used in vivo or that ex vivo activation and expansion is always necessary.

Unprimed T cells can be rapidly activated by CD3xCD19 BsAb

We now had to address the question of whether intra-venously administered BsAb would be self-supporting in activating the T cells in addition to targeting the T cells with BsAb to the tumor cell. If not, additional in vivo stimulation would be necessary. Most T cells from the peripheral blood (PBL-T) have little or no cytotoxic activity. Therefore, PBL-T have been activated ex vivo with mitogens or CD3 mAb in combination with IL-2, loaded with BsAb and used for loco-regional administration in patients with solid tumors (reviewed in reference[39,40,111]). Ex vivo activated T cells exert disadvantageous homing properties. Pre-activated T cells display a strong preference for the lungs and, at later time points, for the liver. They have little capacity to recirculate through lymphoid tissues, in contrast to resting T cells, which traffic preferentially through lymphoid tissue when leaving the blood.[8,10] For hematologic tumors including lymphoma loco-regional administration is not sensible because these tumors are generally disseminated; intravenous administration of BsAb seems to be more logical. To monitor the generation of cytotoxic $CD4^+$ and $CD8^+$ T cells in peripheral blood cell samples, in response to stimulation with the BsAb, short-term cultures were started.[112] Surprisingly, we found activated cytotoxic T cells in the PBMC fractions within 24 hours of CD3xCD19 BsAb stimulation. Cells retained this capacity during a 3-day culture period. Only at day three they needed repeated addition of BsAb to obtain optimal lysis. These results hold true for PBMC from normal donors and NHL patients. Both $CD4^+$ and $CD8^+$ cells exerted this cytotoxicity. Both T cell subpopulations were dependent on monocytes, despite the presence of normal B cells.

The in vivo efficacy of CD3×anti-idiotype BsAb treatment has been demonstrated in murine B cell lymphoma models,[51–53] where monocytes did not seem to contribute significantly to tumor regression.[52] These data suggest that T cell activation can take place, mediated by the number of receptor-coreceptor pairs on T and B cells.[113] The in vivo efficacy of BsAb plus IL-2 therapy was demonstrated in immunocompetent mice with a syngeneic B cell lymphoma. Mice treated with both BsAb and IL-2 showed prolonged survival.[114] Therefore, additional activation of these T cells may be necessary. To establish the influence of IL-2 on BsAb induced T cell-mediated target cell killing, a clonogenic assay was developed.

The effect of BsAb and IL-2 in a clonogenic assay

The efficacy of BsAb, developed to trigger cytotoxic activity of the effector cells towards tumor target cells, is generally measured in a standard 4 h ^{51}Cr-release assay. To measure the cytotoxic capacity of the BsAb, preactivation of the T cells is necessary. To represent the in vivo situation better than this ^{51}Cr-release assay does, a 14-days assay based on limiting-dilution[115,116] was developed to achieve a more sensitive measurement of both the cytostatic and cytolytic capacity of this BsAb in vitro, employing resting PBMC and a clonogenic $CD19^+$ pre-pre-B ALL cell line (REH).[117] With this assay we could evaluate the potency by which T cells from normal donors and NHL patients are targeted and activated by the BsAb to lyse tumor cells in vitro.[118]

This clonogenic assay established the importance of repeated addition of CD3xCD19 BsAb and IL-2. Repeated administration of each of the two agents and the effector to target cell ratio both determined the killing efficacy of BsAb treatment in this assay. Conditions for optimal elimination of the tumor cells (up to 5 log elimination) included: 1) stimulation on day 0, 3 and 6 with CD3xCD19 BsAb (100 ng/ml) in combination with IL-2 (50 U/ml), and 2) the total amount of PBMC mixed with the tumor

target cell resulting in an effector (T cell) to target cell (pre-pre-B ALL cell) ratio of 3:1. Under these conditions, PBMC isolated from NHL patients in remission or relapse are as effective as normal donor PBMC in the elimination of ALL-B cells.

Data obtained with these preliminary experiments might indicate that clinical responses in CD3xCD19 BsAb immunotherapy, will only be obtained in patients with minimal tumor load, and may need additional T cell stimulation via cytokines such as IL-2.

IV administration of CD3xCD19 BsAb; a phase I study

Several monoconal antibodies specific for B cells are available. In contrast to the situation in solid tumors lacking real tumor specific antibodies[54,55,119] systemic application of BsAb in patients with B cell tumor seems less dangerous. In 1992 a phase I study of intravenously administered CD3xCD19 BsAb (SHR-1) was started and three patients which fulfilled the entry criteria have been treated.[120] The BsAb was infused daily over nine days in escalating doses from 10 µg up to 5 mg. The trial showed little toxicity, consisting of moderate fever and chills or shivers (grade 2 WHO), and some remarkable phenomena were observed, although limited results were obtained. Directly after i.v. administration of the BsAb, lymphocytes were found to leave the circulation. Although we could not establish to which site(s) the lymphocytes homed, it is tempting to speculate that they re-located also in lymph nodes. In two patients with LG-NHL transient decrease in lymph oedema was seen which might be due to a decrease in tumor cell mass located in the lymph nodes, resulting in a better lymph flow. In one patient, activated T cells were found in the lymph nodes as detected by immunohistochemistry. A few hours after the administration of BsAb increased serum concentrations of both TNFα and soluble CD8 (sCD8) were detected, which decreased over the next 24 hours. Specifically the detection of a rise of sCD8 might indicate that CD8 positive cells became stimulated. No production of IL-6 was determined, which might indicate that no monocyte or macrophage activation had taken place. Conversely, in the three patients included in this trial there was no indication of damage to the MPS system, as demonstrated by unchanged uptake of Technetium-colloid by liver and spleen, excluding the possibility that T cells kill FcγR positive cells by reversed ADCC. Clearly, much remains to be done before this BsAb is routinely used for therapy, but the results obtained show that the CD3xCD19 BsAb may have a potential as a therapeutic agent in B cell malignancy.

DISCUSSION AND FUTURE DIRECTIONS

Can immunotherapy in B cell malignancy be of value when combination chemotheraoy, radiotherapy or autologous bone marrow transplantation (BMT) finally result in a consistent relapse. While tumor response to conventional monoclonal antibody therapy has generally been limited,[14] the use of bispecific monoclonal antibodies capable of binding both to the effector and target cells continues to hold promise. The aim of the study was to develop CD3xCD19 bispecific monoclonal antibodies, that could be tested for efficacy in vitro and in vivo in patients with B cell malignancy, and to understand and overcome some of the obstacles to achieve clinical success.

Tumor destruction with the help of BsAb is unlikely to succeed in patients without prior ex vivo activation of effector cells or additional in vivo stimulators since unprimed T cells are only minimally cytotoxic to tumor targets. Furthermore, tumor target cells alone appeared unable to induce T cell activation. The latter may have two explanations. First, the degree of CD3 cross-linking via the CD19 antigen molecules on the tumor cell may be suboptimal. Second, the restriction may be caused by insufficient secondary signals. Although binding of CD3 mAb to the TCR/CD3 complex leads to the generation of activation signals (Ca^{2+} mobilization and the expression of the IL-2 receptor[121]) this activation by itself is normally not sufficient to initiate efficient T cell proliferation and cytokine production. Consequently, T lymphocytes have been activated ex vivo by a combination of IL-2 and CD3 mAb and re-injected after being coated with BsAb. IL-2 may also be administered by intravenous injection together with the BsAb.[46,54,56,76] Simultaneous administration of a mAb to a costimulatory antigen on the effector cell may be an alternative. For the latter, several approaches e.g. costimulation via CD28 antigen,[122,123] and the development of trispecific $F(ab')_3$,[26,124] have demonstrated their efficacy in vitro. Additional stimulation may be important since activation of T cells through the TCR/CD3 in the absence of any accessory signals leads not only to suboptimal proliferation and lymphokine production,[102,125,126] it also seems to result in clonal anergy of the stimulated T cells.[125,127] Both IL-2 or CD28 mAb can prevent this state of clonal anergy.[102,125,128–130] Which of these will be most useful in BsAb treatment of patients with B cell malignancies needs more specific experiments.

IL-2 has been widely used in immunotherapy based on its immunostimulatory activities.[4,5,11] IL-2 induces T cell- and NK cell-cytotoxicity and activates B lymphocytes as well as monocytes. When administered to cancer patients, IL-2 demonstrated its capacity to activate lymphocyte functions in a dose dependent manner. However, IL-2 has

not been widely used because of severe toxicity (vascular leak syndrome).[131-133] More recently, IL-2 therapy has been focused on the administration of "low-dose" IL-2 that can be administered for longer time periods. To induce T cell activation by IL-2, T cells should be (pre-)activated to induce the expression of the high affinity IL-2 receptor.[134] Additionally, in vitro, at low concentrations, IL-2 promotes the proliferation of the minor subset (10%) of NK cells that express the high-affinity IL-2 receptors. This provides a favorable therapeutic to toxic ratio.[5,6] In addition, when IL-2 is administered subcutaneously, a more constant serum concentration without peak levels can be obtained,[6,135] resulting in lower toxic effects.[136,137]

Administration of mAb or BsAb with CD28 specificity separately from a CD3xCD19 BsAb will result in T cell activation, leaving the NK cells unaffected. Such combinations have been shown effective in tumor cell killing,[138] and may therefore be a promising approach for in vivo immunotherapy. A possible drawback of such effective T cell stimulation is the resulting cytokine release from the CD4+ T cell subset, which is preferentially stimulated by CD28 mAb.[139,140] To limit the activation to the tumor site, a combination of two BsAb could be used. Both would bind a tumor antigen, but the second antigen specificity would be CD3 in one BsAb and CD28 in the second BsAb (CD3xtumor antigen-1 BsAb and CD28xtumor antigen-2 BsAb).[123,138,141]

A second difference to be mentioned between IL-2 and CD28 costimulation is the preferentially induced proliferation of the T cell subpopulations. Culture of PBMC in the presence of IL-2 causes a predominant growth of CD8+ T cells,[76] which are thought to be the most important cytotoxic T cells. However, it has been noticed that CD4+ T cells are not only as potent as CD8+ T cells in exhibiting BsAb mediated cytotoxicity,[142,143] but they also secrete a large variety of important cytokines.[144,145] Cytokines produced by CD4+ T cells can be important for in vivo tumor destruction because they initiate and maintain a specific anti-tumor response.[146] However, these same cytokines may also be toxic. PBMC cultures stimulated with CD3xCD19 BsAb induced proliferation of CD4+ and CD8+ T cells, whereas PBMC cultures stimulated with CD3xCD19 BsAb and CD28 mAb induced proliferation mainly of CD4+ cells.[122] The expression pattern of CD28 may explain this difference, since 60–95% of CD4+ and only 50% of CD8+ T cell were found to express CD28.[139,140,147]

Our intention with BsAb-mediated immunotherapy is to induce T cell activation at the site of the tumor, i.e. the lymphoid organs. Such tumor dependent T cell activation can in principal be attained at least to some degree, with all combinations mentioned above (BsAb plus low-dose IL-2, BsAb plus CD28 mAb, or BsAb plus CD28xtumor-antigen BsAb). More experimentation is required to determine the most optimal protocol combining efficient T cell activation with minimal toxic side effects.

Cross-linking of the TCR/CD3 complex is a prerequisite for most TCR/CD3 mAb-mediated T cell activation. Since monocytes bear receptors for the Fc-parts of mAb, they are able to indirectly provide this cross-linking function. During this mAb mediated contact, the monocyte also supplies the T cell with cytokines such as IL-1.[34,84,86,148] The heavy chain isotype of the mAb/BsAb is of major influence on CD3 mAb-induced T cell proliferation as various isotypes exhibit significant differences in their capability to bind to the human Fcγ receptors.[85,149] Three main classes of Fcγ receptors (FcγRIa (CD64), FcγRIIa (CD32), and FcγRIIIa (CD16)) have been identified. Several isoforms can be distinguished within each receptor class, and further complexity is introduced by various genetic polymorphisms.[78,90] Indeed, we found that purified T cells were efficiently stimulated by CD3 mAb or CD3xCD19 BsAb in the presence of autologous monocytes, or fibroblasts, transfected with the relevant FcγR. Both ratIgG2bxmouseIgG1 and mIgG1xmIgG2a BsAb interact with the high affinity FcγRIa and hence induce T cell mitogenesis, while the mIgG1xmIgG2b BsAb had only a marginal capacity to trigger T cell mitogenesis. A negative aspect associated with FcγR interaction is the development of the "first-dose" syndrome experienced by patients after initial administration of therapeutic mAb.[80,81,94] The symptoms include fever, chills, diarrhea and vomiting, and have been attributed to elevated levels of circulating cytokines such as IL-6 and TNFα.[80,82,83,94] Treatment with mouse IgG2a CD3 mAb, to prevent transplantation rejection, is accompanied by severe side effects associated with a systemic release of cytokines including IL-2, TNFα, IFNγ and IL-6.[80,81,94,150,151] Both activation of T cells or monocytes, by cross-linking TCR/CD3 or FcγR respectively, result in release of cytokines.[82,83,152] It seems therefore logical that fewer side effects were noticed after administration of CD3 mAb with lower affinity for the FcγR.[81,153] This may be explained by reduced release from either cell-type, since absence of proper Fc-FcγR interaction will not only reduce TCR/CD3 cross-linking but also of FcγR.

In summary, clustering of T cells and monocytes by CD3 and FcγR recognition respectively, may have diverse effects. T cell activation in the periphery may result in large numbers of cytotoxic effector cells which can subsequently be targeted to the tumor cells in lymphoid organs. Some reports, however, claim that mAb activated T cells will not home to these organs.[8,10] Such massive T cell activation will moreover give rise to toxic levels of cy-

tokines from both partner cells. Tumor cell induced T cell activation may therefore be preferred. As discussed above, secondary signals like CD28 mAb or IL-2, may in these protocols replace the role of accessory signals delivered by the monocyte (CD80, IL-1). Another aspect of the discussion derives from the fact that human serum contains a high titer of IgG, the natural ligand for FcγR.[154] Occupation of this receptor would block the binding of therapeutic mAb, thereby preventing the accessory function in T cell activation and the augmented cytokine release. Indeed, in vitro, we found that human IgG blocked rIgG2bxmIgG1 as well as mIgG1xmIgG2a BsAb induced T cell mitogenesis. In a number of reports, however, in vivo applied mAb against B cell antigens[70,155,156] induced cytokine release. This argues at least for some degree of FcγR interaction of these mAb. It is not yet understood how such interactions can occur in the presence of excess natural ligand. A possible explanation may lie in the multimerized presentation of the mAb by the relevant antigen positive cells. Whether this can also function in T cell activation is uncertain.

Clinical trials with mouse antibodies showed these to be quite immunogenic in humans, which effect may interfere with therapy.[14,54] Repeated high dose injections of murine monoclonal antibodies may result in the development of human anti-mouse-Ig antibodies (HAMA). Such responses preclude prolonged or repeated treatment. Consequently, the idea was to produce mAb capable of escaping surveillance by the human immune system. It proved difficult to obtain human antibodies by the conventional route of immortalization of human antibody producing cells. Genetic engineering techniques were subsequently applied to produce partly humanized antibodies e.g. by transplantation of the hypervariable regions of a rat or mouse antibodies into human immunoglobulin genes.[35,85,157-160] With the use of partially humanized antibodies, the production of anti-isotypic antibodies may be avoided, however, the development of anti-idiotypic antibodies is not precluded.

Immunotherapy with mAb/BsAb demands a continuous supply of large amounts of mAb of high purity, and low batch to batch variation. With BsAb these requirements are especially difficult to fulfil, due to heterologous H-L chain pairing.[31] On the long term these problems will be bypassed by genetically engineered single chain BsAb.[35,36] Detailed investigation will be needed to establish their clearance rate and performance in T cell stimulation. Will BsAb be routinely used for therapy in the future? The in vitro results and mice studies, thus far obtained, have demonstrated the considerable potential of BsAb to employ the natural immune system for the treatment of tumors. Attempts to treat tumors will be aided by an increasing understanding of the requirements for effector cell activation, e.g. the significance of adhesion molecules, and tumor target cell susceptibility. In vivo, BsAb show little toxicity and some promising results were obtained. The development of new methods for the preparation of bispecific and chimeric monoclonal antibodies gives prospects for large scale production of pure BsAb.

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